

# Biological Degradation of 2,4,6-Trinitrotoluene

ABRAHAM ESTEVE-NÚÑEZ, ANTONIO CABALLERO, AND JUAN L. RAMOS\*

*Department of Biochemistry and Molecular and Cellular Biology of Plants, Estación Experimental del Zaidín,  
 Consejo Superior de Investigaciones Científicas, Apdo Correos 419, E-18008 Granada, Spain*

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## INTRODUCTION

Life on this planet is based on the continuous cycling of elements. In recent years the massive mobilization of natural resources and the industrial synthesis of chemicals have generated a number of environmental problems as a consequence of the limited incorporation of natural and synthesized molecules into ongoing biological cycles. This is particularly true for xenobiotic compounds, which exhibit structural elements or substituents that are rarely found in natural products. The chemical properties of xenobiotic compounds determine their toxicity, their persistence in the environment, and the manner in which they are degraded by microorganisms. Apart from chloramphenicol, nitropyroluteorin (161), oxypyrronitrin (101), and phidolopin (232), no other natural nitroaromatic compound has been described to date, which probably explains why nitroaromatic compounds are relatively refractory to biological degradation and why xenobiotic compounds are not easily incorporated into biogenic element cycles, with the consequent impact on living organisms and ecosystems.

A number of nitroaromatic compounds such as nitrobenzenes, *o*- and *p*-nitrotoluene, 2,4-dinitrotoluene, and nitrobenzoates are mineralized at a relatively slow pace by microorganisms. These chemicals have multiple applications in the synthesis of polyurethane foams, herbicides, insecticides, pharmaceuticals, and explosives (99), all of which are more recalcitrant than the raw material from which they are synthesized. Comprehensive reviews of catabolic pathways leading to the degradation of nitroaromatic compounds are available (27, 53, 54, 79, 87, 88, 106, 122, 123, 141, 147, 170, 210–212, 243).

The most widely used nitroaromatic compound is 2,4,6-trinitrotoluene (TNT). TNT is more recalcitrant than mono- and dinitrotoluenes mainly because of the symmetric location of the nitro groups on the aromatic ring, an arrangement that limits attack by classic dioxygenase enzymes involved in the microbial metabolism of aromatic compounds (186). Therefore, the chemical structure of TNT influences its biodegrad-

ability. This makes it worth analyzing the mechanism of biodegradation of this molecule in detail.

In this review we first analyze the chemical structure of TNT and then describe how aerobic and anaerobic microorganisms attack it. Certain strains of *Pseudomonas* and of fungi are able to use TNT as a nitrogen source by removing nitrogen under aerobic conditions (30, 63, 119, 223). This seems to involve, in some cases, the removal of nitro groups from the aromatic ring, which may occur via the formation of an intermediate  $\pi$ -Meisenheimer complex (77), and the further reduction of the released nitrite to ammonium, which is incorporated into carbon skeletons. It has also been suggested that hydroxylaminodinitrotoluenes are key metabolites for the release of nitrogen from the TNT ring by *Pseudomonas pseudoalcaligenes* (74), although the stage of metabolism when this occurs and the form in which assimilable nitrogen is made available to the cells are unknown.

A number of reports on the mineralization of TNT by *Phanerochaete chrysosporium* and other fungi that mineralize TNT under ligninolytic conditions are available (47, 107, 129, 151, 164, 183, 194, 196, 219–221, 235–237). Strict anaerobic bacteria of the genera *Clostridium* and *Desulfovibrio* (6) can degrade TNT via triaminotoluene, which might subsequently be metabolized to methylphloroglucinol and *p*-cresol, although conclusive biochemical evidence for this pathway is still needed (54, 60, 64, 80, 142, 180). It has also been suggested that some *Clostridium* strains reduce TNT to the corresponding hydroxylaminodinitrotoluenes, which, upon Bamberger rearrangement, yield aminophenols (111, 113). Another pathway has been described in *Pseudomonas* sp. strain JLR11 and involves nitrite release and further reduction to ammonium, with almost 85% of the nitrogen of TNT incorporated as organic nitrogen in the cells (68, 69). In this review we intend to highlight the recent discovery of TNT as an alternative electron acceptor in respiratory chains of a facultative *Pseudomonas* strain (69). We also discuss a number of biotechnological applications of bacteria and fungi, including slurry reactors, composting, and land farming, to remove TNT from polluted soils. These treatments have been designed to achieve either mineralization or reduction of TNT, as well as immobilization of its amino derivatives on humic material. These approaches

\* Corresponding author. Mailing address: Estación Experimental del Zaidín, C/Profesor Albareda 1, E-18008 Granada, Spain. Phone: 34-958-121011. Fax: 34-958-129600. E-mail: jlramos@eez.csic.es.

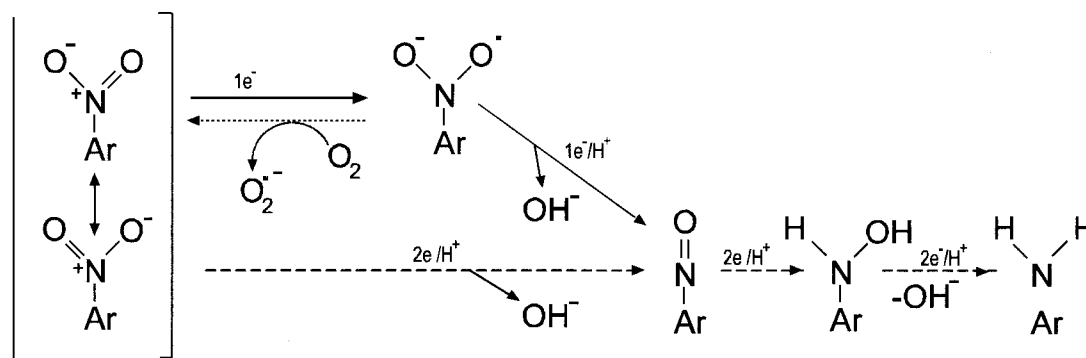


FIG. 1. Mechanisms for the reduction of nitro groups in nitroaromatic compounds. The first step in nitro group reduction can be achieved through one-electron transfer (solid line) or two-electron transfer (dashed line). The first mechanism produces a nitroanion radical that could react with oxygen to form a superoxide radical and the original nitroaromatic compound through a futile cycle (dotted line). If the mechanism occurs via the transfer of two electrons, the nitroso derivative formed is the first putative intermediate; following two consecutive electron transfers, a hydroxylamine and an aromatic amine are produced. The scheme has been adapted from reference 212.

are highly efficient in removing TNT, and increasing amounts of research into the potential usefulness of phytoremediation, rhizophytoremediation, and transgenic plants with bacterial genes for TNT removal are being done.

### CHEMISTRY OF TNT

The  $\pi$  electrons from the aromatic ring of TNT are removed by the electronegative nitro groups, a process that makes the nucleus electrophilic. The nitro group consists of two different elements, N and O, which are both highly electronegative. Oxygen is even more electronegative than the nitrogen atom; hence, the N-O bond is polarized. The partially positive charge of the nitrogen atom, combined with its high electronegativity, makes the nitro group easily reducible (175). Reduction of nitro groups on aromatic rings is widely distributed among living organisms.

In vitro assays showed that in cell extracts prepared from a number of microorganisms and higher eukaryotes, reduction of the nitro group occurs as a series of two-electron transfers which yield the nitroso, hydroxylamino, and amino derivatives of the parent compound (Fig. 1). This reduction is referred to as oxygen independent (type I), and no radicals are formed (45). Nitroreductase enzymes able to perform this reaction use pairs of electrons donated by reduced pyridine nucleotides (10, 25, 43, 44–46, 86, 98, 130–132, 145, 159, 162, 184, 209, 231, 244, 249, 258, 261–263). Other enzymes that reduce nitroaromatic compounds include aldehyde oxidase (254), dihydrolipic amide dehydrogenase (228), cytochrome  $b_5$  reductase (148), diaphorases (126), hydrogenases (58, 149), xanthine oxidase (46), and CO dehydrogenase (111). The nitro group can also be reduced in vitro through single-electron transfers, which form a nitroanion radical (Fig. 1). This radical is a putative intermediate in the reduction of a nitro group to a nitroso group, but it can react with oxygen to produce a superoxide anion and alter the original nitroaromatic compound (Fig. 1). The enzymes that catalyze this reaction are known as oxygen sensitive (type II) nitroreductases and are found in bacteria such as *Clostridium* (9) and *Escherichia coli* (171).

The nitroso and hydroxylamino groups, responsible for the toxicity of nitroaromatic compounds, react with biological mol-

ecules (49) and cause chemical mutagenesis and carcinogenesis (16, 20, 36, 51, 85, 110, 178, 189, 213, 224, 226, 234, 255, 260). Complete reduction of the nitro group to an amino group seems to decrease the mutagenic effect of the compound (50, 82, 227). Hydroxylaminodinitrotoluenes also cause hemotoxic symptoms in workers exposed to TNT, since oxyhemoglobin is oxidized by molecular oxygen in the presence of hydroxylaminodinitrotoluenes to yield ferrihemoglobin and nitrosodinitrotoluenes (15, 52, 128, 144, 177). In blood cells this reaction becomes a futile cycle because nitrosodinitrotoluenes are reduced back to hydroxylaminodinitrotoluenes, which again react with oxyhemoglobin.

Abiotic reduction of the nitro groups to the corresponding amines also occurs in sediments, soils, and aquifers (91, 93). Numerous potential electron donors are present in natural systems (e.g., reduced iron species, reduced sulfur species, and natural organic matter) that may reduce nitroaromatic compounds in abiotic reactions (84, 109, 133, 249). TNT also reacts with the siloxane surface of clays to yield covalent complexes (92, 246–248). The roles that living organisms play in these biogeochemical processes are of interest, but this is still a relatively unexplored area of research (108, 109, 192). The behavior of TNT in soil will be examined in depth in “Applications in TNT bioremediation” (below).

In the reduction of the nitro groups of TNT, the reduction of the first nitro group is generally much more rapid than that of the rest. The conversion of nitro to amino groups decreases the electron deficiency of the nitroaromatic ring, and consequently a lower redox potential is required to reduce the rest of the nitro group of the molecule. Because of this, the formation of triaminotoluene requires values below  $-200$  mV, which are found only in anoxic environments (81, 109). Barrows et al. (17) hypothesized that the step that establishes regioselectivity in the abiotic reduction process is the first proton transfer to the radical anion that is created after the initial one-electron reduction and that the nitro group with the greater negative charge localization is likely to be the one most readily protonated. In TNT, the *para* position is much more easily reduced than the *ortho* nitro groups.

As Averill (13) pointed out, “the nitro group reduction of nitroaromatic compounds ( $\text{ArNO}_2$ ) by bacteria generally pro-

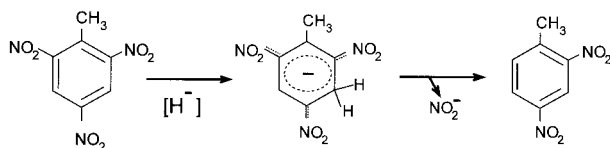


FIG. 2. TNT-Meisenheimer complex formation. The hydride ion can be donated by NAD(P)H, giving rise to a Meisenheimer complex. Aromaticity is restored on the release of a nitro group.

ceeds in two-electron processes such as the reduction of nitrate during denitrification". One might therefore expect the chemical processes in the reduction of  $\text{NO}_2^-$  and  $\text{ArNO}_2$  to be analogous, since both the inorganic and the organic species contain nitrogen in the same oxidation state (+3) and two N-O bonds; however, the arrangement of the valence electron on the nitrogen atom, especially the absence of lone pairs, makes  $\text{ArNO}_2$  more similar to nitrate ( $\text{NO}_3^-$ ) than to nitrite ( $\text{NO}_2^-$ ).

The deficiency of electrons in the aromatic nucleus of TNT makes nucleophilic attack on the ring possible; as a result, a nonaromatic structure such as a Meisenheimer  $\sigma$  complex can be formed (70, 125, 229). These compounds are red-orange and have a negative charge (Fig. 2). Such complexes can be formed *in vivo* by hydride attack from a reduced pyridine nucleotide. The Meisenheimer complex can be rearomatized after nitrite release, with the formation of dinitrotoluenes. Oxygen is not necessary for the formation of this kind of compound, and so this process is an alternative for nitroaromatic metabolism when oxygenic removal of the nitro groups is not possible, as in TNT.

#### AEROBIC METABOLISM OF TNT BY BACTERIA

The aim of this section is to review the best-known mechanisms of aerobic metabolism of TNT by bacteria (Fig. 3). Oxygenolytic metabolism of the nitro groups of nitroaromatic compounds is limited to mononitroaromatic and dinitroaromatic compounds (8, 37, 95–97, 105, 155, 156, 158, 182, 191, 211, 214, 217, 225, 261). Oxygenolytic metabolism for aromatic compounds by bacteria does not occur in TNT because of its chemical properties, as described above. Therefore, even in the presence of oxygen, this explosive must be transformed by reductive metabolism.

In most of the cases described to date, aerobic bacteria tend to transform the TNT molecule by reducing one or two nitro groups to hydroxylamino or amino groups and to produce different isomers of aminonitroaromatic compounds, which in turn usually accumulate in the culture medium without further metabolism. It has also been reported that partially reduced forms of TNT react among themselves in the presence of oxygen to form recalcitrant azoxytetranitrotoluenes (94), which causes a higher rate of mutations than does TNT (82). These transformation reactions remove TNT but yield highly recalcitrant products that are not metabolizable by most of the microorganisms that produce them (16, 36, 110, 118, 215, 226).

Only a few aerobes able to use TNT as a nitrogen or carbon source have been reported, and mineralization of this compound has been described even less frequently (Table 1). Elimination of the nitro group is required to decrease the electro-

philic nature of the molecule and allow dioxygenases to use di- or mononitroaromatic compounds as suitable substrates. This fact led researchers to isolate microorganisms able to use TNT as the sole nitrogen source in mineral medium supplemented with additional carbon sources (30, 63, 119, 233). None of these researchers reported TNT mineralization, because the different strains they isolated produced very low levels of  $^{14}\text{CO}_2$  from [ $^{14}\text{C}$ ]TNT.

Duque et al. (63) isolated a *Pseudomonas* strain from soil around an explosives factory and found that it was able to grow with TNT as the sole nitrogen source. Nitrite accumulated in culture supernatants, and the sequential removal of all three nitro groups was suggested based on the identification of dinitrotoluenes, mononitrotoluenes, and toluene in the culture supernatants. The elimination of TNT by this *Pseudomonas* strain was proposed to occur via the production of a Meisenheimer complex (94, 179). This kind of complex was also reported by many authors as a metabolite involved in polynitroaromatic metabolism by bacteria (77, 185, 242). Lenke and Knackmuss (139) isolated a *Rhodococcus erythropolis* strain able to use 2,4,6-trinitrophenol (picric acid) as a nitrogen source after initial reduction of the aromatic ring by a hydride ion (186). Vorbeck et al. (242) suggested that a similar hydrogenation reaction of the aromatic ring of TNT also occurred in resting cells of *R. erythropolis*, although the strain failed to use TNT as nitrogen source, probably because the corresponding TNT complex undergoes neither nitrite elimination nor rearomatization to 2,4- or 2,6-dinitrotoluene under physiological conditions (138, 242). This route of dihydride complex formation is therefore unproductive for the catabolism of TNT in *Rhodococcus* spp. In contrast, Haïdour and Ramos (94) reported the formation of 2,4-dinitrotoluene (2,4-DNT) as a result of the *in vitro* metabolism of the TNT-Meisenheimer complex by *Pseudomonas* clone A (Fig. 3); however, no stoichiometric analyses were done by these authors, and this reaction could not be confirmed by Vorbeck et al. (242).

Degradation of TNT to 2,4-DNT is desirable, since previous work has shown that other *Pseudomonas* strains can metabolize this nitroaromatic through dioxygenase attack to produce 4-methyl-5-nitrocatechol and that this is followed by oxidation, release of nitrite, and mineralization (96, 155, 158, 214).

*Enterobacter cloacae* strain PB2, originally isolated for its ability to use nitrate esters, can also slowly grow with TNT as the sole source of nitrogen. French et al. (77) obtained *in vitro* evidence for the reduction of TNT with purified pentaerythritol tetranitrate (PETN) reductase and showed that this enzyme reduced TNT to its hydride Meisenheimer complex with concomitant NADPH oxidation and release of nitrite. Because hydroxylaminodinitrotoluene and aminodinitrotoluene were detected, the enzyme was also assumed to have other nitroreductase activities. The gene encoding PETN reductase, designated *onr* (for "organic nitrate reductase"), has been cloned and overexpressed in *E. coli*. A recombinant *E. coli* strain that expressed PETN reductase was also able to release nitrite from TNT. This is the first report of the reduction of the aromatic ring of TNT by a purified enzyme with the concomitant release of nitrite from TNT. A biotechnological application of this enzyme was recently reported (75) and will be discussed below (see "Applications in TNT bioremediation"). Recently, Pak et al. (162) have reported the purification of an NADH-depen-

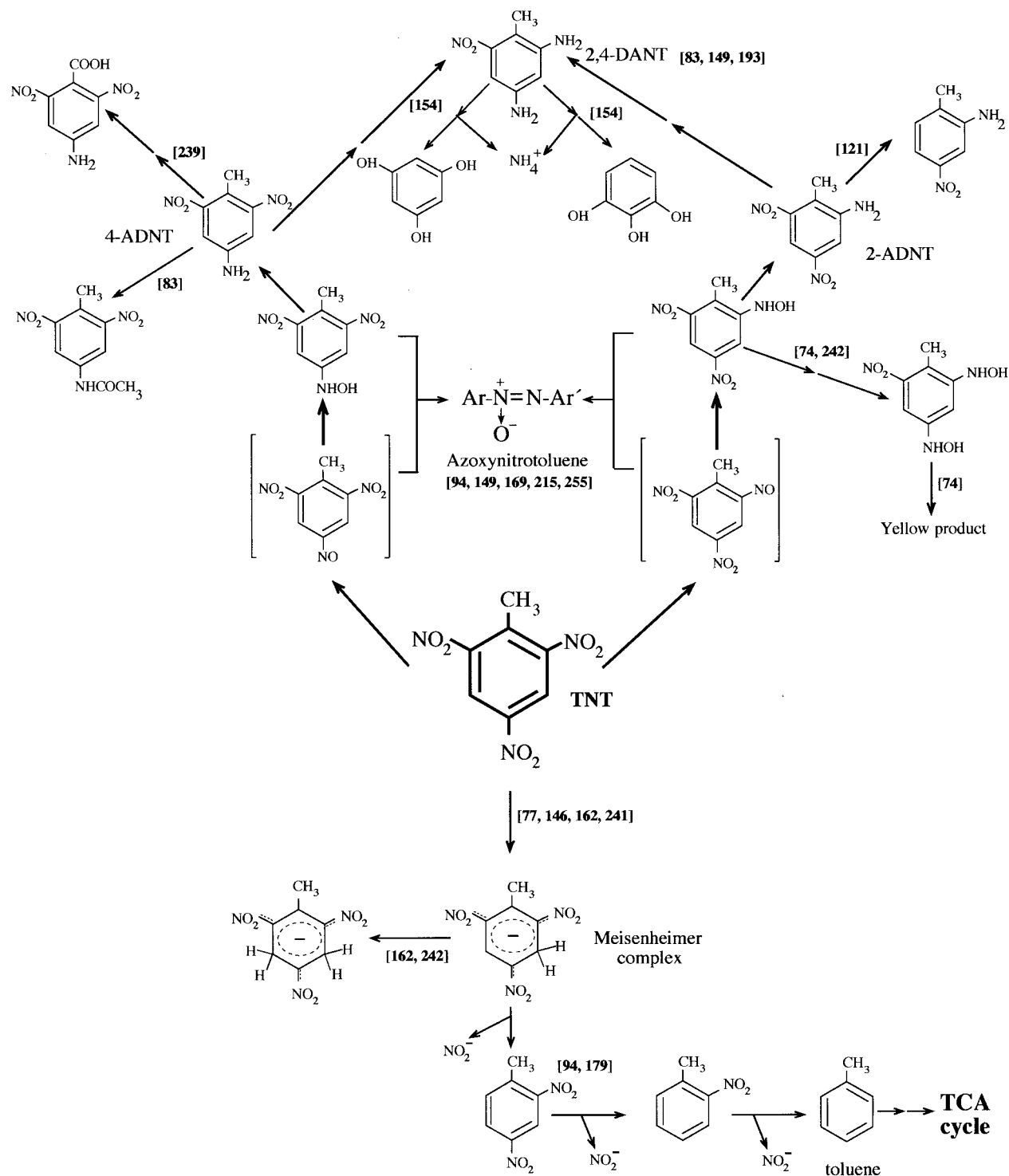


FIG. 3. Pathways for the aerobic metabolism of TNT in aerobic microorganisms. The scheme is based on articles cited in the text, which are indicated in the figure in square brackets. When the conversion of a compound to another is believed to occur through a series of intermediates, the steps are indicated by two arrows. TCA, trichloroacetic acid.

dent flavoprotein oxidoreductase (XenB) (26) from *Pseudomonas fluorescens* which catalyzed the reduction of TNT by adding a hydride to the aromatic ring with the concomitant formation of dihydride Meisenheimer complex or catalyzed the reduction of nitro groups.

Other authors also reported denitration reactions in TNT. Martin et al. (146) reported such a reaction in a *Pseudomonas savattoni* strain unable to mineralize significant quantities of TNT (less than 1%) but able to transform TNT into 2,4-DNT and nitrite. This reaction seems to be enhanced by removing



TABLE 1. Microorganisms reported to degrade or transform TNT under aerobic conditions

Microorganism	Metabolism	Reference(s)
<i>Bacillus</i> sp.	Nitrite release from TNT and transformation to 2-amino-4-nitrotoluene	121
<i>Enterobacter cloacae</i> PB2	TNT reduction to a Meisenheimer complex with nitrite release	77
<i>Enterobacter</i> sp.	TNT transformation to unknown polar compounds and 3% mineralization	14
<i>Mycobacterium vaccae</i>	50% radioactivity from [ <sup>14</sup> C]TNT incorporated into lipids	239
<i>Mycobacterium</i> sp. strain HL4NT-1	Cells grown in 4-nitrotoluene transform TNT to hydride and dihydride Meisenheimer complex	241, 242
<i>Pseudomonas aeruginosa</i>	Nitrite release from TNT and transformation to 2-amino-4-nitrotoluene	121
<i>Pseudomonas aeruginosa</i> MA101	Transforms TNT and ADNTs into highly polar compounds through an O <sub>2</sub> -dependent process	7
<i>Pseudomonas fluorescens</i>	An NADPH flavoprotein oxidoreductase catalyzes multiple transformations, generating ArNHOH, H-TNT and H <sub>2</sub> -TNT	162
<i>Pseudomonas fluorescens</i> B3468	TNT degradation to phloroglucinol and pyrogallol; nitrogen of TNT is released as ammonium	154
<i>Pseudomonas fluorescens</i>	TNT transformation into ADNT, DANTs, and 4-acetamide-2-amino-6-nitrotoluene	83
<i>Pseudomonas pseudoalcaligenes</i>	Reduction of nitro groups and nitrite release from 2,4-dihydroxylamino-6-nitrotoluene	74
<i>Pseudomonas savastanoi</i>	Denitration of TNT to release nitrite and reduction of nitro group to amino	146
<i>Pseudomonas</i> sp. clone A	Strain able to denitrate TNT	63
<i>Pseudomonas</i> sp. strain CBS3	Reduces TNT to DANT	193
<i>Pseudomonas</i> sp. strain FR2	Reduces TNT to DANT	149
<i>Pseudomonas</i> sp. strain IIBx	First evidence of [ <sup>14</sup> C]TNT mineralization	233
<i>Pseudomonas</i> sp. strain 1-2wt	First evidence of [ <sup>14</sup> C]TNT mineralization	233
<i>Pseudomonas</i> sp.	TNT used as sole nitrogen source; TNT transformation to 2ADNT and 4ADNT	119
<i>Rhodococcus erythropolis</i>	Cells growing on picric acid catalyze ring hydrogenation, forming hydride and dihydride TNT-Meisenheimer complexes	242
<i>Serratia marcescens</i>	TNT as sole carbon and energy source in presence of Tween 80	153
<i>Staphylococcus</i> sp.	Nitrite release from TNT and transformation to 2-amino-4-nitrotoluene	121
<i>Stenotrophomonas maltophilia</i>	TNT as sole nitrogen source	160

ammonium and adding nitrite to the growth medium. In contrast, the addition of glucose enhanced 2-amino-4,6-dinitrotoluene (2-ADNT) and 4-amino-2,6-dinitrotoluene (4-ADNT) production and decreased the denitration of TNT.

Recently, Kalafut et al. (121) reported a promising aerobic route for TNT metabolism which led to the production of 2-amino-4-nitrotoluene. This metabolite results from the loss of one of the original nitro groups, leaving two adjacent unsubstituted carbon atoms that can serve as a substrate for oxygenases. This compound was detected in cultures of three different strains of aerobic bacteria (*Pseudomonas aeruginosa*, *Bacillus* sp., and *Staphylococcus* sp.). However, no further details are known.

Bae et al. (14) reported the transformation of TNT to polar products by a strain of *Enterobacter*. These authors found 3% mineralization, conversion of 80% of the TNT to unidentified polar products, and conversion of 13% to a biomass-associated fraction. Changes in the nature of the TNT molecule leading to increased polarity may indicate hydroxylation or cleavage of the aromatic ring. Such transformations may facilitate further metabolism by other organisms, but characterization of the unknown products is necessary.

Vanderberg et al. (239) isolated a *Mycobacterium vaccae* strain that was able to metabolize TNT in the presence of propane as the carbon and energy source. Usual TNT reduction metabolites were found, but two novel oxidized metabolites were generated during TNT catabolism: aminodinitrobenzoic acid and diamino-nitrobenzylmethyl ether. The latter compound seems to be a dead-end product, and the authors proposed the *o*-methylation of the corresponding benzylalcohol as a mechanism of detoxification. Although mineralization

was not observed, a substantial portion of the radioactivity from ring-labeled TNT (ca. 50%) was incorporated into the cellular lipid fraction. These results suggest that ring cleavage occurred prior to the incorporation of radiolabeled carbon into polar lipids. Because of its potential interest for biodegradation, further studies of the ring cleavage process in this strain are expected.

The reduction of nitro group compounds to amino compounds has been described as a gratuitous reaction, whereas partial reduction of nitroaromatic compounds to hydroxylamino derivatives has been identified as a key reaction in productive catabolic routes (90, 156). Fiorella and Spain (74) proposed a mechanism of partial reduction of TNT on the basis of their work with cells and cell extracts of nitrobenzene-grown *P. pseudoalcaligenes* (Fig. 3). These authors identified 2-hydroxylamino-4,6-dinitrotoluene and 2,4-dihydroxy-6-nitrotoluene; the latter seems to be produced by nitrobenzene reductase, and in the presence of oxygen it was converted into an unidentified yellow compound (Fig. 3). The release of nitrogen from the above aromatic compounds has not yet been explained, but the authors suggested that it may occur via the release of nitrite (small amounts were detected). Vorbeck et al. (242) also focused on hydroxylaminoaromatic compounds as key intermediates that make the nitrogen in TNT available for growth. TNT nitrogen users were found to convert TNT to 2-hydroxylamino-4,6-dinitrotoluene and 4-hydroxylamino-2,6-dinitrotoluene, together with a more polar compound than the latter ones. The authors suggested that these strains obtained N for growth from the above-cited hydroxylaminonitrotoluenes, although it is unclear at which stage of metabolism and in which form assimilable nitrogen was made available to the

TABLE 2. Microorganisms reported to anoxically degrade or transform TNT

Microorganism	Metabolism	Reference(s)
<i>Clostridium acetobutylicum</i>	Reduction of TNT to TAT	64, 127
<i>Clostridium bifermentans</i> CYS-1	Degrades TNT to aliphatic polar compounds via 4ADNT and 2,4DANT	205
<i>Clostridium bifermentans</i> LJP-1	Transforms TNT into TAT and phenolic compounds	141
<i>Clostridium pasterianum</i>	Reduction of TNT to TAT	149
<i>Clostridium sordelii</i>	Reduction of TNT to TAT	64
<i>Clostridium</i> sp.	Bamberger rearrangement of dihydroxylaminodinitrotoluene	113
<i>Desulfovibrio</i> sp. strain B	TNT as nitrogen source, toluene as a putative intermediate	29
<i>Desulfovibrio</i> sp.	TNT as the sole nitrogen source; reduction of TNT to TAT	174
<i>Desulfovibrio</i> sp.	Transforms TNT into TAT and DANT; 42% of radioactivity from [ <sup>14</sup> C]TNT is associated with cell biomass	60
<i>Escherichia coli</i>	Reduction of TNT to TAT	64
<i>Lactobacillus</i> sp.	Reduction of TNT to TAT	64
<i>Methanococcus</i> sp. strain B	Reduction of TNT to DANT	30
<i>Pseudomonas</i> sp. strain JLR11	TNT as nitrogen source; TNT as final electron acceptor	68, 69
<i>Veillonella alkalescens</i>	Reduction of TNT to TAT	149

bacterial cells. The removal of N from the TNT ring through hydroxylamine aromatic compounds or via the formation of Meisenheimer complexes seems to explain the aerobic mechanism of TNT degradation by bacteria.

The unspecific reduction of TNT to amino derivatives, which usually accumulate, is a widely distributed reaction among aerobic bacteria (Fig. 3). Only a few reports have examined aerobic metabolism of these aminoaromatic compounds by microorganisms (7, 83, 154). Alvarez et al. (7) reported a strain of *P. aeruginosa* that aerobically metabolized aminodinitrotoluenes (ADNTs) to highly polar compounds which were not extractable with organic solvents. This transformation was oxygen dependent, and the products were not identified. No mineralization was detected, and the authors suggested that oxidative metabolism of the ADNTs might occur through oxidation of the methyl group and its subsequent decarboxylation or through the action of oxidative deaminases. Naumova et al. (154) cultured a strain of *Pseudomonas fluorescens* by using the partially reduced metabolites ADNTs as the nitrogen source. Incubation of cell extracts from cultures grown on TNT transformed DANT into phloroglucinol and pyrogallol, and 30% of the DANT nitrogen was released as ammonium. In spite of this activity in vitro, the microorganism was not able to use DANTs as a source of carbon and energy, and the physiological significance of this reaction in vivo remains to be clarified.

#### ANAEROBIC METABOLISM OF TNT BY BACTERIA

Anaerobic processes have the potential advantages of rapid reduction at low redox potential, which minimize oxidative polymerization of substrates because of the absence of oxygen. Anaerobic systems for TNT degradation have therefore been studied to attain more efficient rates of removal, since azoxy-nitrotoluene products are not formed (91, 94). Microorganisms reported to anoxically degrade or transform TNT are listed in Table 2.

The earliest evidence of the anaerobic metabolism of TNT was reported by McCormick et al. (149), who showed that cell suspensions or crude extracts of *Veillonella alkalescens* could reduce TNT to triaminotoluene (TAT), with H<sub>2</sub> as an electron donor (Fig. 4). In the last 10 years several reports have shown

that this reduction is possible under anaerobic conditions because the low potential required for TAT makes this reaction impossible in aerobic microorganisms (60, 64, 80, 174).

Two genera have been extensively studied because of their anoxic metabolism of TNT: *Clostridium* and *Desulfovibrio*. The ability to reduce TNT anaerobically is a general phenomenon among *Clostridium* species (64). In cell suspension assays with clostridia, reduction of the nitro group could result in the production of TAT and other products, some of which remain unidentified. The question whether the pathways for TNT metabolism are inducible or associated with constitutively expressed metabolic functions in different *Clostridium* strains remains to be solved (61, 64). Below we describe some in vivo and in vitro reactions of TNT by *Clostridium* spp. Crawford and colleagues (80, 142, 180) reported cometabolic TNT transformation by *Clostridium* strains isolated from a long-term bioreactor fed with explosives (TNT, trihydroxytoluene trimethylenetrinitronitramine [RDX], tetramethylenetetranitramine [HMX]), sewage sludge, and soils. TAT was identified in these cultures, and aromatic compounds lacking nitrogen substitution (methylphloroglucinol and *p*-cresol [Fig. 4]) were detected (54), together with phenolic products from TAT hydrolysis (142). These authors showed that the addition of a cosubstrate that supplied reducing power for nitro group reduction was required for significant TNT degradation (205). Shen et al. (202) have proposed that the *p*-cresol detected during the anaerobic metabolism of TNT might originate from the metabolism of some aromatic amino acids such as tryptophan rather than from TNT.

Hughes et al. (113), working with cell extracts of *Clostridium* strains, detected the formation of 2-amino-4-hydroxylamino-5-hydroxyl-6-nitrotoluene and 2-hydroxylamino-4-amino-5-hydroxyl-6-nitrotoluene (Fig. 4), which may have arisen from Bamberger rearrangement of dihydroxydinitrotoluene. Recently Huang et al. (111) have suggested that the initial reaction could be mediated by CO dehydrogenase and that this enzyme may transform TNT into 2-hydroxylamine-4,6-dinitrotoluene, 4-hydroxylamine-2,6-dinitrotoluene, and 2,4-dihydroxylamine-6-nitrotoluene as reduction products. Via Bamberger rearrangement, the last compound may subsequently yield 2-amino-4-hydroxylamino-5-hydroxyl-6-nitrotoluene and hydroxylamine-4-amino-5-hydroxyl-6-nitrotoluene. Subsequently, Ahmad and Hughes

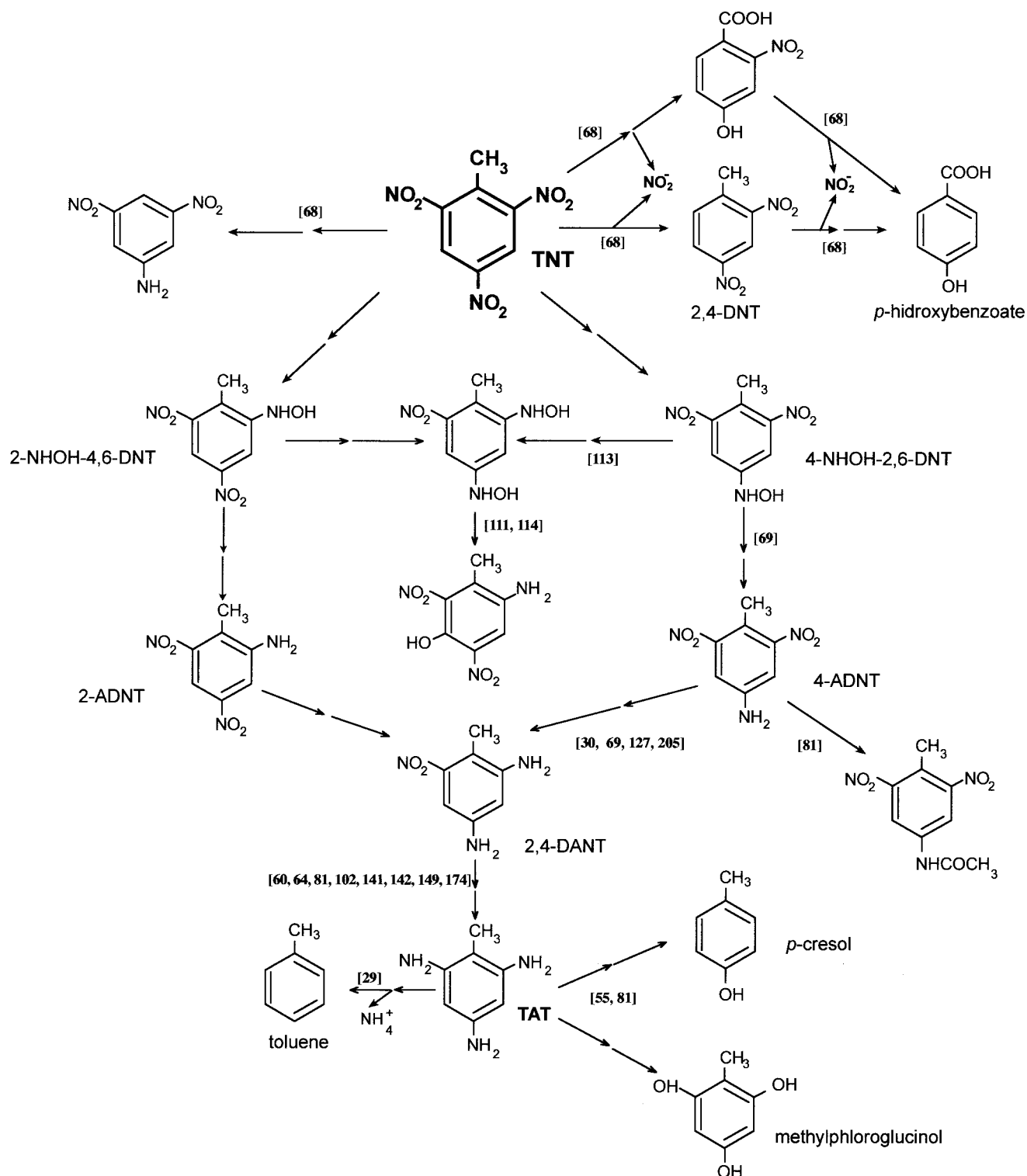


FIG. 4. Proposed mechanisms for anaerobic TNT metabolism in bacteria. The scheme is based on articles cited in the text, which are indicated in the figure in square brackets. When the conversion of a compound to another is believed to occur through a series of intermediates, the steps are indicated by two arrows.

(6) suggested that the incorporation of an aerobic stage to cleave aminophenols formed in clostridial systems could help to mineralize nitroaromatic contaminants. In some *Clostridium* strains, dihydroxylamino intermediates seem to play an impor-

tant role not just in TNT but also in DNT metabolism (115). However, further metabolism of dihydroxylaminotoluenes resulted in the formation of arylamines and not in aminophenols as in the dihydroxylaminonitrotoluene rearrangement (113).

*Desulfovibrio* strains have been also extensively studied because of their ability to metabolize TNT under anoxic conditions. Boopathy and Kulpa (29) isolated a strain, called *Desulfovibrio* sp. strain B, that used TNT as the sole nitrogen source and accumulated toluene in the culture medium (Fig. 4). The authors hypothesized that the nitro group was reduced to the corresponding amines to form TAT, which then underwent reductive elimination of the amino groups by a mechanism analogous to that described by Schnell and Schink (198) for aniline. The proposal was preliminary, since the authors failed to detect TAT or any evidence of deamination.

Preuss et al. (174) isolated a pure culture of *Desulfovibrio* which was selected by using TNT as the sole nitrogen source and pyruvate as the carbon and energy source. This strain was able to catalyze the complete reduction of TNT to TAT in cell suspensions in the absence of any reducing agent. The authors suggested that the reduction of DANTs to TAT was the rate-determining step in the overall process. No reductively deaminating reaction was found, and no toluene was detected in cell suspensions of the isolate. The authors also carried out biochemical studies of the microbial enzymes that catalyze TNT reduction. The kinetics of DANT reduction by *Desulfovibrio* spp. depended on the electron donor. With pyruvate and H<sub>2</sub>, DANTs was converted to TAT; in contrast, the addition of carbon monoxide (CO) inhibited the production of TAT, and 2,4-diamino-6-hydroxylaminotoluene accumulated in the medium. These observations led the authors to believe that sulfite reductase was responsible for the reduction of 2,4-diamino-6-hydroxylaminotoluene to TAT because this enzyme is known to be inhibited by CO. However, assays with purified enzymes have not yet been done.

Drzyzga et al. (60) described biotransformation assays with [<sup>14</sup>C]TNT in *Desulfovibrio* strains and found that although the explosive was not mineralized, 42% of the initial radiolabel was associated with the cell biomass. TAT and both DANT isomers were detected in liquid supernatants.

When TNT degradation by *Clostridium* and *Desulfovibrio* spp. seems to proceed via TAT, the mineralization of TNT under anaerobic conditions must involve the elimination of the amino groups from TAT. The biological transformation of TAT is possible only at neutral pH and under anoxic conditions; otherwise the molecule is unstable (175). However, the role of TAT as the key metabolite in the anaerobic metabolism of TNT has been questioned by Hawari et al. (103), who suggested that this compound is a dead-end product which prevents mineralization of the nitroaromatic. The chemical properties of TAT in an anaerobic atmosphere were used by Rieger and Knackmuss (185) to design a bioremediation technique (see "Applications in TNT bioremediation" below) which consists of full reduction of the explosive to TAT using anaerobic incubations with sewage sludge cultures.

The anaerobic removal of TNT under different electron-accepting conditions (nitrate, sulfate, and carbon dioxide) has been reported. Nitrate respiration conditions gave the best degradation rate with a consortium of soil bacteria (33), whereas methanogenesis seemed to be optimal with slurries of aquifer sediment (135).

Ederer et al. (64) tested anaerobic degradation of TNT by enterobacteria (*E. coli* and *Salmonella enterica* serovar Typhimurium) and lactobacilli (*Lactobacillus acidophilus*, *L. casei*,

and *L. latiss*) and found that these species were able to reduce TNT but not DANT.

More recently, Ramos and colleagues described a different mechanism for the anoxic degradation of TNT (68, 69). They isolated a facultative *Pseudomonas* sp., designated strain JLR11, which was able to grow on TNT as the sole nitrogen source with glucose as a cosubstrate. Mass balance assays with TNT revealed that about 85% of the total nitrogen in the nitroaromatic compound was incorporated as cell biomass. Analysis of the culture supernatants detected pathway intermediates lacking nitro groups, e.g., 2-nitro-4-hydroxybenzoic acid, 4-hydroxybenzaldehyde, and 4-hydroxybenzoic acid (Fig. 4). The conversion of TNT to these compounds requires not only the removal of nitro groups but also oxidation of the lateral methyl group of TNT to the corresponding aldehyde and carboxylic acid. In assays with [<sup>14</sup>C]TNT, only 1% of the radioactivity was detected as <sup>14</sup>CO<sub>2</sub> but 45% was associated to trichloroacetic acid-precipitable cell material. The productive initial attack on the nitroaromatic compound seemed to involve nitrite release, since nitrite accumulated transiently with TNT consumption. Cell extracts of *Pseudomonas* sp. strain JLR11 grown anoxically in TNT showed nitrite reductase activity (69). A mutant selected after *mini*-Tn5-tellurite mutagenesis as being unable to grow on TNT as the sole nitrogen source had also lost the capacity to grow on nitrite as the sole nitrogen source (67). Cell suspension assays with the mutant in a TNT medium resulted in the release of nitrite, which accumulated with time. This accumulation was not observed in identical experiments using the wild-type strain.

Strain JLR11 reduced a fraction of the total TNT to ADNTs, and these products accumulated with time and were not used by the strain as a nitrogen source. The authors determined that the reduced forms of TNT are produced by *Pseudomonas* sp. strain JLR11, because TNT acted as a final electron acceptor. To verify this, strain JLR11 was cultured with TNT and acetate as a cosubstrate; under these conditions the oxidation of acetate under anoxic conditions required an electron acceptor, a role that only TNT could play in this assay. Proton translocation, together with the reduction of TNT, was detected in anoxic *Pseudomonas* sp. strain JLR11 cell suspensions cultured in medium containing TNT, but this extrusion did not occur when nitrite- or nitrate-grown cells were incubated with TNT.

Esteve-Núñez et al. (69) also found synthesis of ATP coupled to H<sub>2</sub>-TNT oxidation-reduction in membrane vesicles prepared from JLR11 cells cultured with TNT as the final electron acceptor (Fig. 5). When nitrate or nitrite replaced TNT as the final electron acceptor, the rates of ATP synthesis were on the order of 10 and 35%, respectively, of the rate in the presence of TNT. The role of TNT as an electron acceptor was suggested previously by Boopathy and Kulpa (29), although Esteve-Núñez et al. (69) demonstrated experimentally that the reduction of TNT in *Pseudomonas* sp. strain JLR11 was linked to proton extrusion, which may contribute to a transmembrane electrochemical proton gradient of sufficient magnitude to drive ATP synthesis. The physiological role of TNT respiration raises the possibility of interesting environmental applications in anaerobic environments polluted with TNT, in which the pollutant might be used not only as a nitrogen source but also as a terminal electron acceptor.



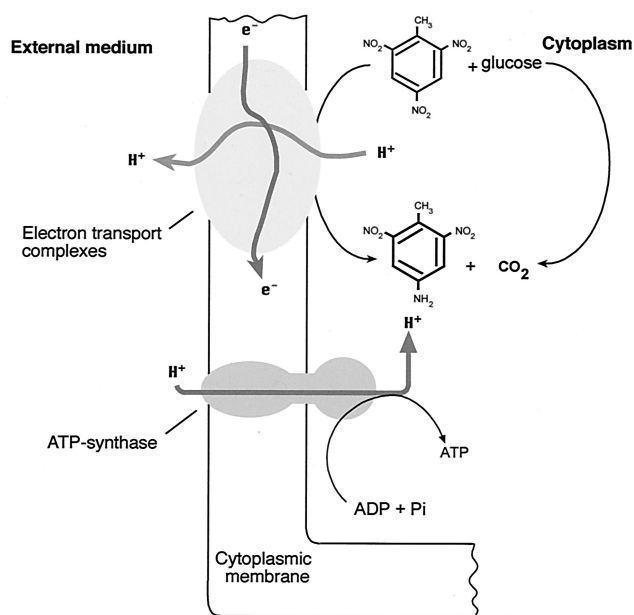


FIG. 5. Scheme showing the coupling of electron donor compounds, TNT oxidoreduction, and ATP synthesis. Pi, inorganic phosphate. The scheme has been adapted from reference (69).

### FUNGAL METABOLISM OF TNT

The use of fungi for the bioremediation of TNT has generated considerable interest in the last decade. The wood white rot fungus *Phanerochaete chrysosporium* has been the subject of intensive study, and more recently other white rot fungi and litter decay fungi have been investigated for their ability to transform TNT.

The interest in white rot fungi arises from their capacity to degrade a diverse group of environmentally persistent and toxic chemicals. Early observations revealed TNT metabolism by *P. chrysosporium* under nitrogen-limiting conditions (47, 72, 102). Later it was shown that different nutrient starvation conditions (carbon, nitrogen, or sulfur limitation) favoured TNT and other xenobiotic attack and that these reactions were carried out by secreted enzymes of the lignin-degrading system. This system contains lignin peroxidase, manganese peroxidase (MnP), oxidases, reductases, hydrogen peroxidase, veratryl alcohol, oxalate, and quinol oxidases (221).

As in other organisms, the initial steps in the fungal degradation of TNT involve the reduction of nitro groups (164). Mycelia of *P. chrysosporium* reduce TNT to a mixture of 4-ADNT, 2-ADNT, 4-hydroxylamino-2,6-dinitrotoluene, and azoxytetranitrotoluenes (Fig. 6). Under ligninolytic conditions the aromatic compounds and azoxytetranitrotoluenes disappear, and mineralization can be fairly extensive.

Stahl and Aust (219–221) provided evidence that TNT is reduced by a plasma membrane redox system in *P. chrysosporium* that requires live and intact mycelia. Any conditions that disrupt the integrity of the plasma membrane destroy the reductase activity. The presence of compounds known to inhibit the membrane redox systems also inhibits TNT reductase. The authors suggested that reduction is coupled to the proton export system used by the fungus to maintain the extracellular

physiological pH at approximately 4.5. In contrast, Rieble et al. (183) reported a membrane-associated TNT reductase activity that required NADPH as a cosubstrate and the absence of molecular oxygen. Because activity was detected in the detergent-solubilized form, where a membrane potential could not be maintained, a membrane potential redox system was assumed to be inessential for this reaction. On the other hand, NAD(P)H-dependent intracellular TNT reductase activity has also been described (151).

Regardless of the site where TNT is reduced, further degradation of these compounds and eventual mineralization of TNT by *P. chrysosporium* occur only when cultures are ligninolytic, implying that lignin peroxidase, MnP, and/or other enzymes of the lignolytic system further transforms the reduced products of TNT. The mechanism by which the fungi mineralize the explosive is not known, but preliminary information about the process has been reported (107, 151). When ligninolytic cultures of *P. chrysosporium* were incubated with 4-ADNT, a compound identified as 4-formamido-2,6-DNT was detected (Fig. 6). This intermediate was transformed into 2-amino-4-formamido-6-nitrotoluene, a compound that disappeared rapidly under ligninolytic conditions but not under non-ligninolytic ones. In nonligninolytic environments the ADNT metabolites were slowly reduced to DANTs and the concentration of azoxytetranitrotoluenes increased (Fig. 6).

The hydroxylaminodinitrotoluene products of TNT reduction inhibit the veratryl alcohol oxidase activity of lignin peroxidase (47, 150). This activity protects lignin peroxidase from  $H_2O_2$  inactivation, and explains why the presence of the hydroxylaminodinitrotoluenes makes ADNT more readily mineralizable than TNT (221). The design of bioremediation processes for TNT-contaminated soils with *P. chrysosporium* therefore requires conditions under which hydroxylaminodinitrotoluenes do not accumulate.

The degradation of azoxytetranitrotoluenes by *P. chrysosporium* is not unexpected, because the reduction of azoxy compounds to azo compounds is chemically simple and the mineralization of various azo dyes by this fungus has been demonstrated (165). The catabolic potential of *P. chrysosporium* for TNT mineralization is limited because growth of this fungus is inhibited at relatively low concentrations of TNT and because reduction of this polynitroaromatic compound by immobilized *P. chrysosporium* mycelia is inhibited above 20 ppm. The low tolerance of *P. chrysosporium* to TNT prevents this fungus from being a good candidate for bioremediation of TNT-contaminated sites, which usually show a high concentration of this nitroaromatic compound.

Other fungi also exhibit potential for TNT degradation (129). In this regard it was reported that the MnP of several white rot fungus attacks TNT (194–196). Scheibner and Hofrichter (194) showed that cell-free preparations of MnP from the white rot basidiomycete *Nematoloma frowardii* and from the litter-decaying basidiomycete *Stropharia rugosoannulata* were able to mineralize a mixture of reduction products from  $[^{14}C]$ TNT as well as 2-amino-4,6- $[^{14}C]$ dinitrotoluene (195, 196). More recently, Van Aken et al. (237, 238) showed that a concentrated preparation of MnP from a different white rot fungus, *Phlebia radiata*, was able to completely transform TNT (22% mineralization) and 2-amino-4,6-dinitrotoluene (76%



## APPLICATIONS IN TNT BIOREMEDIATION

The manufacture, processing, and packaging of TNT at ammunition plants for several decades have resulted in high concentrations of contaminants in soil (168, 199, 257), and eventually this pollutant reaches the groundwater (18, 169, 206). A number of physicochemical treatments have been proposed, and some of them are in use (190). Currently, incineration is the most effective remediation alternative, but it is expensive for use with polluted soils because of the costs of soil excavation, transport, and energy for incineration. For the treatment of TNT in liquid wastes, several technologies have been proposed, e.g., chemical reduction (5, 11, 12, 66, 137), thermal decomposition, subcritical water (104), photocatalytic degradation (197), electrohydraulic discharge (250), granular iron (59), and ozonolysis (136, 216, 222). Several biologically based technologies have also been developed for the remediation of TNT-contaminated soils; of these approaches, the most thoroughly investigated are soil slurry reactors, composting, and land farming. Each approach has advantages and disadvantages, and the main applications are summarized below. Many groups have developed bench-scale assays, but only a few have tried full-scale assays (80, 140).

Soil slurry technologies consist of reactors filled with a mixture of soil and water to which cosubstrates and nutrients can be added as necessary. These systems are designed to optimize mass transfer of nutrients and electron acceptors by using mechanical mixing and/or aeration. All work with this technology has used anoxic incubation to avoid the polymerization reactions that take place in the presence of oxygen; sometimes an additional aerobic treatment is required. The metabolism of TNT in anoxic processes requires an additional cosubstrate (e.g., starch, glucose, sucrose, or molasses), which plays two main roles: oxygen removal by growing aerobes, and electron donation for nitro group reduction of TNT. There are two different approaches to TNT bioremediation by slurry reactors: mineralization of the explosive as the main target (31, 32, 80, 81) and irreversible binding of TNT metabolites to the soil matrix (140, 178, 185). The method of Funk et al. (80, 81), developed, patented, and licensed at the University of Idaho, appears to be useful for the biological remediation of soils contaminated with nitroaromatic compounds and explosives. This procedure was previously used for the strictly anaerobic microbial bioremediation of nitroaromatic-herbicide-contaminated soils (188). The process, designated SABRE (sequential anaerobic biological remediation *ex situ*), consists of a consortium of facultative anaerobic organisms that transform explosives such as TNT to nontoxic, nonaromatic, and aerobically mineralizable products. The consortium contained strains of the genus *Clostridium*, and spores of these strains are expected to be useful as inoculants for bioremediation (200).

The accumulation of starchy material in treated soil produces a high oxygen demand, which may be detrimental in agricultural soils because of the rapid development of anaerobic conditions when the soil is wetted, such as after irrigation or rainfall. Roberts et al. (187) proposed an aerobic treatment after the anoxic stage to remove excess external carbon and obtain treated soil with a lower oxygen demand. The process they reported required glucose as a cosubstrate and the presence in the soil of a population of nitroaromatic-degrading

anaerobes (80, 81). Nishino et al. (157) reported that the addition of specific nitroaromatic-mineralizing bacteria seemed to enhance the mineralization of dinitrotoluenes in soil slurries whereas native bacteria did not transform the compound during the incubation time required for the mineralization of DNTs.

The U.S. Army Environmental Center has developed a bio-slurry system to provide a nonproprietary process for the treatment of explosives-contaminated soil (117). The process is similar to the SABRE and was conducted in a trench reactor. The results of analytical and toxicity tests support the potential usefulness of land applications of the processed water, although an additional aerobic treatment seems to be necessary to bring down the biological oxygen demand of the treated waters.

Boopathy et al. (28) did a laboratory study using a soil slurry reactor with a dissolved-oxygen profile which made the reactor to function as an aerobic-anaerobic system. TAT was removed in the batch mode during the first 2 weeks; however, reduction metabolites persisted and semicontinuous operation over a period of 3 months was recommended by the authors. TNT degradation was demonstrated by observing mineralization of [ $^{14}\text{C}$ ]TNT (23% of total  $^{14}\text{C}$  evolved as  $^{14}\text{CO}_2$ ) and by finding 27% of the radioactivity as trichloroacetic acid-precipitable material.

The soil slurry reactor technology has also been used to enhance the anaerobic fixation of xenobiotic compounds to the soil organic matrix, a process mediated by microbial cometabolic activities (185, 223). Bench-scale assays have been developed, and all of them showed complete TNT removal and incorporation of 84 to 99% radioactivity ([ $^{14}\text{C}$ ]TNT) to the soil matrix (56, 60, 61) after anoxic-aerobic treatment. This biologically induced immobilization of TNT was tested on a technical scale (140) in a Terranox reactor with 18 m<sup>3</sup> of TNT-contaminated soil (314 mg of TNT/g of soil) mixed with 10 m<sup>3</sup> of water supplied with sucrose and ammonium chloride. The process led to removal of 98% of the TNT, DNT, and RDX in 30 weeks. The decrease in the concentrations of the explosives was slower than in the laboratory assay, possibly because of the lower temperature and mass transfer as a result of less intense mixing. The extraction of bioremediated soils with various chemicals did not lead to the release of TNT or its metabolites (1). Binding seems to increase with the number of amino groups in the aromatic ring, making TNT undetectable in the supernatants. The results of the ecotoxicological evaluation of the supernatant after anaerobic incubation showed toxic effects, but no toxicity was detectable after the subsequent aerobic treatment. Terrestrial tests performed at the end of the bioremediation process showed no mortality for earthworms.

Composting is considered a feasible technology for the remediation of munitions-contaminated soils. Research thus far indicates that TNT can be removed from contaminated soils (35, 42, 61, 62, 124, 167, 253). In composting, the soil must be mixed with a bulk agent and additional organic substrates which are usually by-products of agricultural processes (straw, alfalfa, wood chips, sugarbeet stems, and leaves). Windrow composting and anaerobic-aerobic composting systems are highly efficient for treatment of TNT-contaminated soils (42). Under these conditions the explosive was reduced to amino- and diammonitrotoluenes during the anoxic phase, and the

subsequent aerated phase eliminated most of the transformation products, possibly by covalent binding to the soil (39, 40, 134). In tests of the toxic effects of these compounds on human monocytes, leachates of bioremediated soil produced a response similar to that of compost-free of nitroaromatic compounds (41). Tan et al. (227) reported that composting of TNT markedly reduced mutagenicity in the contaminated soil, and this was confirmed by others (89, 116). The main criticisms of the compost technique are the long incubation time, the cost of setting up and maintaining the system, and the lack of knowledge about the bacteria and fungi involved in the process.

Land farming is a solid-phase treatment process in which contaminated soil is mixed with nutrients and moisture, using periodical mechanical turning of the soil mixture to increase aeration (71, 117, 250). Widrig et al. (250) investigated an innovative approach to land farming for the bioremediation of TNT-contaminated soil using molasses as a cosubstrate and shredded grass to improve soil management properties. Degradation of TNT was demonstrated by mineralization of [ $^{14}\text{C}$ ]TNT and the presence of  $^{14}\text{C}$  in the cell biomass as trichloroacetic acid-precipitable material. The results of this bench-scale study are promising with regard to transferring the process to full-scale applications. Due to the solubility of TNT and its derivatives in water, hydraulic control of infiltrated water may be required in the design of any on-site or in situ method.

The ubiquitous reductive metabolism of TNT present in all treatments describe above yields large amounts of hydroxylaminonitrotoluenes and ADNTs. Most of the reduction products bind strongly to the mineral (56, 109) and organic (1–4, 38, 56, 57, 60–62, 65, 143) fractions of the soil. After treatment, the reduction metabolites cannot be desorbed from soil by alkaline or acidic hydrolysis or by methanolic extraction, so the authors concluded that metabolites become unavailable for further microbial degradation and mineralization. The covalent binding of reduced metabolites of [ $^{15}\text{N}$ ]TNT to soil organic matter was analyzed by  $^{15}\text{N}$  nuclear magnetic resonance (NMR) spectroscopy (1, 134, 173); the NMR shifts showed that nitrogen was covalently bound to humic acid as substituted amines and amides, whereas the NMR spectra of silylated humin suggested the formation of azoxy compounds and imine linkages. This indicates immobilization of the aminoaromatic compounds to the soil matrix and can be considered a detoxification process, although information about the long-term fate of these bound metabolites is not available. Recently, Sheremata and Hawari (204) reported finding a natural surfactant able to desorb TNT, aminonitrotoluenes, and diaminonitrotoluenes from polluted soils.

Phytoremediation seems to be another alternative for the in situ treatment of explosives-contaminated soils. Recent reports have described assays with a variety of plant systems such as yellow nutsedge (163), bush bean (100), switchgrass (172), aquatic and wetland species (21, 22, 23, 48, 112, 166, 240), axenic root cultures (112), and hybrid poplar (230). In most studies, TNT transformation by plants was accompanied by the appearance of its monoamino derivatives 2- and 4-ADNT. Little is known about the product profile of TNT biocatalysis of plants beyond these early products. Some plants seem to sequester or integrate TNT metabolites into biomass as conjugates (201, 245). The formation of such conjugates of organic

xenobiotic compounds is an important protective phase in plant detoxification metabolism. Some of these conjugates are compartmentalized into plant organelles such as the vacuoles. The primary reduction products of TNT seem to be involved in such conjugation processes (24).

Plant-bacterium combinations to phytoremediate contaminated soil are also being developed (207) with a *Pseudomonas* strain capable of transforming TNT to its monodinitrotoluene and diaminonitrotoluene metabolites. Inoculation of meadow brome grass (*Bromus erectus*) with this strain increased the portion of the rhizosphere community involved in nitroaromatic metabolism and led to a reduction in soil TNT levels.

More recently, transgenic plants that express microbial degradative enzymes for TNT bioremediation have been developed (75). Transgenic tobacco plants that carry the *onr* gene, which encodes the pentaerythritol tetranitrate reductase from *Enterobacter cloacae* (76, 77), showed detectable expression of the reductase enzyme in leaf and root tissue. Transgenic plants that express the explosives-degrading enzyme are able to germinate and grow in the presence of explosives concentrations inhibitory to wild-type plants. Further research is required to determine whether transgenic plants are capable of contributing to the degradation of TNT.

#### PERSPECTIVES IN TNT DEGRADATION AND UNEXPLORED RESEARCH

TNT degradation has attracted the attention of many scientists in the last decade, probably because a number of Environmental Protection Agencies have declared it a pollutant whose removal is a priority. The symmetrical positioning of the nitro groups prevents the attack of classical dioxygenases which generate hydroxylated aromatic rings that subsequently become the substrates of ring cleavage enzymes. In contrast, reduction of the nitro groups seems to be a widely distributed reaction among aerobic and anaerobic bacteria and fungi. However, there are not many details about the biochemical properties of these TNT reductases or the genes that encode them, and we expect many important contributions in this research area. Many anaerobic bacteria and fungi are able to reduce TNT. The derivatives of this reduction are further metabolized and eventually mineralized. In contrast, when these products are produced by aerobic bacteria, they usually constitute dead-end products. The productive steps leading to the mineralization of reduced TNT derivatives by anaerobic bacteria and fungi are unknown, and advances are envisaged by using microorganisms susceptible to the combination genetic and biochemical analyses. These studies will be illuminating not only in terms of understanding the catabolic pathways themselves but also in attempts to gain insights into the evolution of these activities in bacteria and fungi. The wide distribution of TNT-degrading microbes and the abundance of catabolic pathways that can lead to mineralization of TNT lead us to envisage that interest in the field will be focused on the optimization of the catabolic properties of these microbes rather than on the development of recombinant strains, as suggested previously (179), as an alternative to TNT degradation.

A number of laboratories have shown that TNT denitration can yield many different products depending on the microorganism under scrutiny. Except for the reaction catalyzed by



pentaerythritol tetranitrate reductase on TNT, little, if anything, is known about these denitrase activities. We have recently cloned the denitrase cluster of *Pseudomonas* sp. strain JLR11 as a means of giving *Burkholderia cepacia* R34 the ability to grow on TNT. An in-depth analysis of this gene cluster may shed light on the mechanism underlying the reaction. In turn, gene probes can be useful for studying the distribution of this set of genes in nitroaromatic-polluted soils. The use of gene probes will be useful in identifying niches in which these kinds of genes prevail and the conditions under which the population of microbes bearing these genes increases (251). This may also be useful in the design of biological treatments for sites polluted with TNT. Rhizoremediation of TNT by microbes capable of colonizing the rhizospheres of plants will provide a cheap, fast, and efficient process for the removal of this pollutant from the upper layers of the soil.

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